UNIVERSITY OF CALIFORNIA, SAN DIEGO

Interleukin-33: Role in asthma and a promising inhibitor

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by

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The thesis of Jenna-Marie Emilia Magat is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Interleukin-33: Role in asthma and a promising inhibitor

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Jenna-Marie Emilia Magat

Master of Science in Biology

University of California, San Diego, 2013

Professor Timothy Bigby, Chair
Professor Michael David, Co-Chair

Asthma is a disease of the airways for which the pathogenesis is still unknown. Recently, the Interleukin-1 (IL-1) family pair, IL-33 and its receptor ST2, has been implicated in asthma. IL-33 activates both innate and adaptive immune cells and induces the release of T_H2 cytokines. IL-33 signals through the adaptor protein, MyD88, which forms complexes with interleukin receptor associated kinases (IRAK) 4 and 2 that auto-phosphorylate and activate mitogen-activated proteins kinases (MAPK) and nuclear

factor-κB (NF- κB) proteins. To further explore the mechanisms of IL-33, we developed a unique mouse model using IL-33 to replicate an allergic asthma-like phenotype independent of antigen and the adaptive immune system. Next, we wanted to determine if this IL-33 induced asthma-like phenotype could be inhibited. The Rebek laboratory of the Scripps Research Institute synthesized α-helical mimetics of an IRAK2 domain critical for protein-protein interaction (PPI) between IRAK4 and IRAK2. We found that the lead α-helical mimetic was able to decrease IL-33 induced NF- κB activity *in vitro*. Mice treated with the lead α-helical mimetic and challenged with IL-33 had attenuated airway hyperresponsiveness and airway inflammation compared to controls. This shows that using α-helical mimetics to block IL-33 signaling is a possible strategy for asthma therapy. Conclusively, the IL-33 pathway is a promising target in treating asthma, making further understanding of its mechanisms a necessity.

I:

Introduction

Asthma

Asthma is a chronic lung disease characterized by airway hyperresponsiveness and inflammation and has clinical symptoms such as shortness of breath, wheezing, coughing, chest tightening, and mucus production. Although these downstream effects of asthma have been well defined, the pathogenesis of asthma remains a poorly understood. For this reason, even after over 40 years of intensive research, asthma therapy is only moderately effective and often costly. Asthma is a heterogeneous disease, and in order to find treatments, the origins and factors attributing to its development must be properly understood. Recently, the role of genetic and environmental influences on asthma has garnered more attention. Genome-wide association studies show genes involved in barrier function and innate pathways, such as *ORMDL3, IL33 and SMAD3*, are closely linked with asthma susceptibility (1). Levels of exposure to pathogens may also effect the development of asthma, particularly in susceptible subjects. The "Hygiene Hypothesis" (2) proposes that urbanization has changed the mucosal microbiota and in turn the immune system is not getting properly challenged in early life. The immune system may respond incorrectly later on triggering inflammatory responses, allergy, and autoimmunity (3, 4). Eighty-90% of asthmatics have evidence of allergy such as elevated IgE, suggesting that asthma is allergically mediated (5) and thereby linked to the adaptive immune system. Asthma was also shown to be driven by the cytokines IL-4, IL-5, and IL-13 that are typically associated with T helper type 2 (T_H2) cells. As a result, the research has focused on determining how the adaptive immune system initiates T_H2 immunity to drive the asthma phenotype. However, current research shows that the

innate system can also induce T_H2 cytokines. The innate immune response is fully encoded in the germline DNA and uses receptors that recognize molecular patterns of microbes or endogenous danger signals. On the other hand, the adaptive immune system utilizes gene rearrangement and somatic hypermuation to form antigen-specific receptors after exposure to a new antigen. Consequently, the innate immune system is much faster and can activate inflammatory pathways that may work with or independently of the adaptive immune system. Current data also shows that therapies targeting T_H2 cytokines in asthma have only had limited success. Signs point to the innate immune system as a key player in the pathogenesis of asthma and hopefully greater scientific understanding of its mechanisms will lead to clinical breakthroughs.

Innate Immunity

The innate immune system is particularly important in the lungs as they are constantly subjected to environmental exposure. The respiratory environment is a fine-tuned system in which both the cellular and humoral elements work together to provide defense against invading microbes. The overall objectives of the innate immune system are to: detect microbes, initiate responses to eradicate microbes, and activate the adaptive immune system (6). The airway epithelium is home to a multitude of innate mechanisms that participate in protection from infection. Structurally, airway epithelial cells use cilia and tight junctions as a barrier to microbes. Dendritic cells cross tight junctions to extend dendrites out to the external environment and capture antigens (7). Airway epithelial cells also secrete antimicrobial mucus which contains mucins, collectins, and defensins (8). Detection of microbes largely occurs through pattern recognitions receptors (PRRs)

such as Toll-like receptors and NOD-like receptors, which recognize molecules that are highly conserved among microbes, called microbe associated molecular patterns (MAMPs) or peptides released by damaged cells, called damage associated molecular patterns (DAMPs). Pattern recognition receptors are expressed by a range of different cells including non-professional immune cells, such as the lung epithelium, to professional immune cells, such as macrophages and dendritic cells (9). Activation of these receptors initiates their respective signaling cascades that cause transcriptional upregulation of genes encoding various cytokines, chemokines, and antimicrobial peptides. Many of these pathways activate nuclear factor-κB (NF-κB),a transcription factor complex for hundreds of proinflammatory genes (10). The production of these inflammatory mediators then direct innate immune cells to carry out powerful inflammatory responses. Macrophages respond by phagocytosing microbes as well as aiding in the recruitment of other myeloid cells. Basophils, neutrophils, and eosinophils can destroy microbes by degranulation, which releases cytotoxic molecules. Eosinophils are increased during parasitic infections and allergic immune responses. Interleukin-5 has been shown to promote eosinophils in numerous ways by extending cell survival, acting as a chemotactic agent, improving cell adhesion, and boosting effector functions (11). In addition to activating eosinophils and alveolar macrophages, IL-13 was found to cause airway remodeling and airway hyperresponsiveness although the mechanisms are still being examined (12). A transition to the adaptive immune system is initiated simultaneously and is mainly carried out by professional antigen presenting cells, which include mature dendritic cells and macrophages. The initial innate immune response participates in the regulation of the type of adaptive immune response induced. Adaptive

immunity is tailored to the infection and is generally characterized by the subset of helper T cells that are activated and their respective cytokine profile. Specifically, Th1 responses are activated by intracellular bacteria, T_H2 responses are induced by parasites, and T_H17 responses are caused by extracellular bacteria and fungi (13). In addition, memory B cells are also activated to produce antibodies. Overall, innate immunity is highly effective in handling microbial invasions; however, if any part of the system is malfunctioning, balance is disrupted and is very hard to restore. Improper firing of the immune system can lead to cytokine overproduction that has many detrimental effects, such as septic shock. The increased and abnormal expression of T_H2 cytokines are widely accepted as a lead cause of asthma (14); therefore it is imperative to pinpoint which parts of the innate immune system are responsible for the development of this phenotype.

Toll-Like Receptors

One of the most important mechanisms by which the innate system senses microbial invaders is through the Toll-like receptors (TLRs). Named for its homology to the Toll receptor discovered in the fruit fly *Drosophila melongaster* in 1996 (15), the Toll-like receptor family has grown to 10 human receptors, with mice expressing TLR 1-9 homologs. The TLRs are type I transmembrane proteins, consisting of an ectodomain with leucine rich repeats (LRRs), a single transmembrane helix, and a highly conserved cytoplasmic signaling domain (16). The signaling domains of the TLR family are known as Toll/IL-1 receptor (TIR) domains since they are homologous to the signaling domains of the IL-1 receptor family (17), forming the TIR domain superfamily (Fig. 1). Each

TLR can recognize a unique ligand or set of ligands associated with bacteria, fungi, protozoa, or viruses. Toll-like receptors are designated to specific membranes based on what type of ligand is detected and are found on the cell surface, phagocytosed to lysosomes, or within endosomes. When a TLR binds a ligand, dimerization occurs and is thought to cause an important conformational change between the two TIR domains of the TLRs. Adapter proteins containing TIR domains can then associate with the TLR receptor complex by forming TIR:TIR domain interactions. Different signaling cascades are activated depending on which of the 5 adapter proteins get recruited, from "MyD88, TIR domain-containing adaptor inducing IFN-β (TRIF; also known as TICAM-1), TIRAP/Mal, TRIF-related adaptor molecule (TRAM), and Sterile-alpha and Armadillo motif-containing protein (SARM)" (9). Toll-like receptor 4 is stimulated by lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls. When activated, TLR4 forms a homodimer and recruits adaptor protein MyD88, which leads to the release of pro-inflammatory cytokines TNF, IL-1, and IL-6. These cytokines can cause various responses including fever, hypotension, inadequate tissue perfusion, metabolic acidosis, and organ failure . Several studies have also shown links between LPS and pulmonary diseases, including asthma (18). Importantly, the triggering of TLRs by exogenous antigens in the airways often influences which type of downstream innate immune responses are activated.

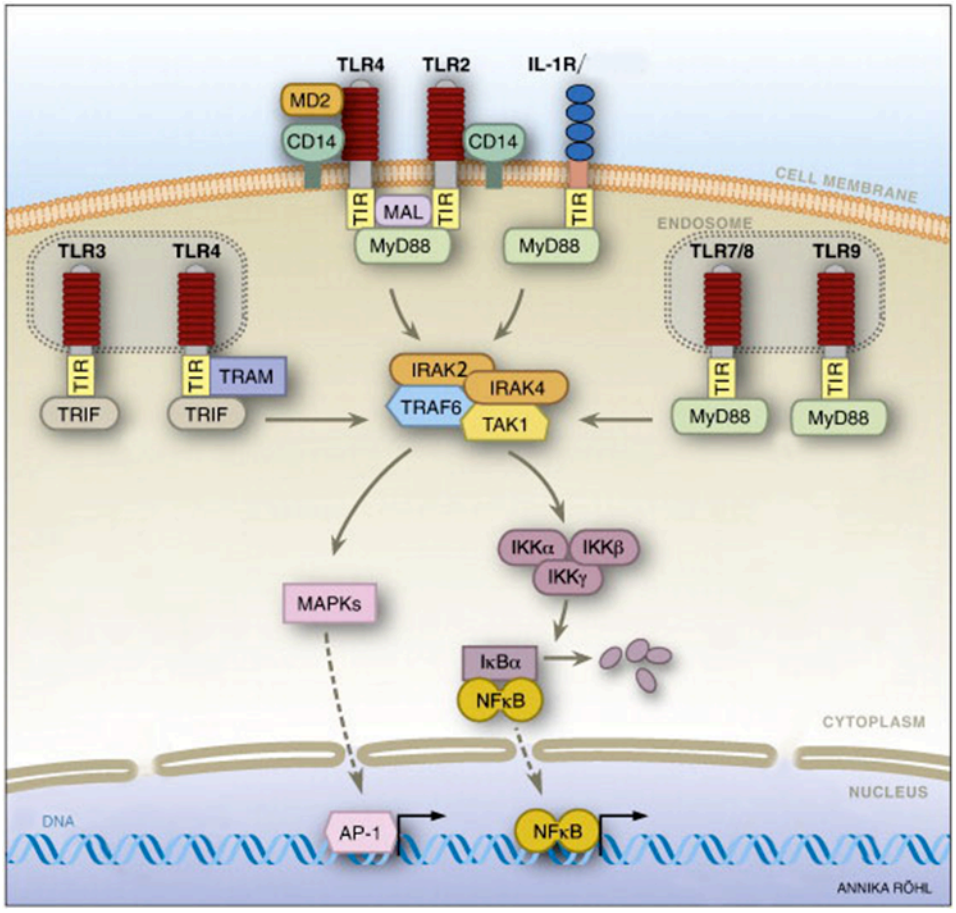

Figure 1: TLR/IL-1R signaling pathway. Binding of MyD88 at the TIR domains of the TLRs or IL-1Rs initiates recruitment of IRAKs and activation of TRAF6 and TAK1, ultimately triggering NF-κB and p38 MAPK pathways (Figure modified from Lundberg, et al. 2009).

Interleukin-1 Receptor Family:

The IL-1 family of cytokines and receptors play a central role in the innate immune system as potent mediators of inflammation and directors of the adaptive immune response. The IL-1R family is an evolutionarily later development of the innate immune system, often called a "modified TLR" (6). Interleukin-1 receptors have an extracellular immunoglobulin (Ig) domain, a transmembrane domain, and a cytosolic TIR domain. Binding of ligand to the primary receptor allows for the recruitment of a second receptor subunit, resulting in a heterodimer. Similarly to TLRs, the repositioning of the

TIR domains recruits the adaptor protein MyD88 and allows for activation of downstream inflammatory pathways including mitogen activated protein kinase (MAPK) and NF-κB (19). The IL-1 family consists of 11 cytokines which are ligands for the IL-1 Receptor family (20). Toll-like receptor signaling often drives the production of members of the IL-1 family of cytokines. TheIL-1 family are endogenous activators, which can be secreted by innate cells and act directly on them as well. Interleukin-1 family cytokines drive specific adaptive immune responses by differentiating naïve T cells into mature helper T cell subsets. Interleukin-18 polarizes T_H1 cells, IL-33 polarizes T_H2 cells, and IL-1 polarizes T_H17 cells and increases B cell proliferation (21). These cytokines generally work extracellularly, although IL-1α (22) and IL-33 (23) have nucleotide binding motifs and appear to function as transcriptional repressors binding to heterochromatin. IL-1β and IL-18 are cleaved into a biologically active form by a multiprotein complex called the inflammasome (24). IL-1 is a key member of the IL-1 family and has two forms, IL-1α and IL-1β, which both signal through IL-1R1/IL-1R accessory protein (IL-1RAP) complex (21). In particular, IL-1β is a proinflammatory cytokine and was initially made known for its ability to induce fever (25). IL-1β is now known to play a role in a variety or acute and chronic inflammatory diseases including rheumatoid arthritis and type-2 diabetes. Also, IL-1β is implicated in asthma for its ability to induce airway hyperresponsiveness through the MAPK pathway (26). In concert, patients with asthma have elevated IL-1β levels in their airways (27). Mainly produced by monocytes and macrophages, IL-1β induction can be triggered by a myriad of different activators including IL-1 itself (28). To counteract the powerful nature of IL-1, receptor agonists are upregulated when IL-1 is induced. Interleukin-1 receptor agontist

(IL-1Ra) binds IL-1R to block receptor signaling (29), and IL-1R2 (30) binds IL-1 as a decoy receptor. Additionally, single immunoglobulin IL-1R-related molecule (SIGIRR), an IL-R family member, dampens TIR domain signaling by obstructing adaptor protein association (31).

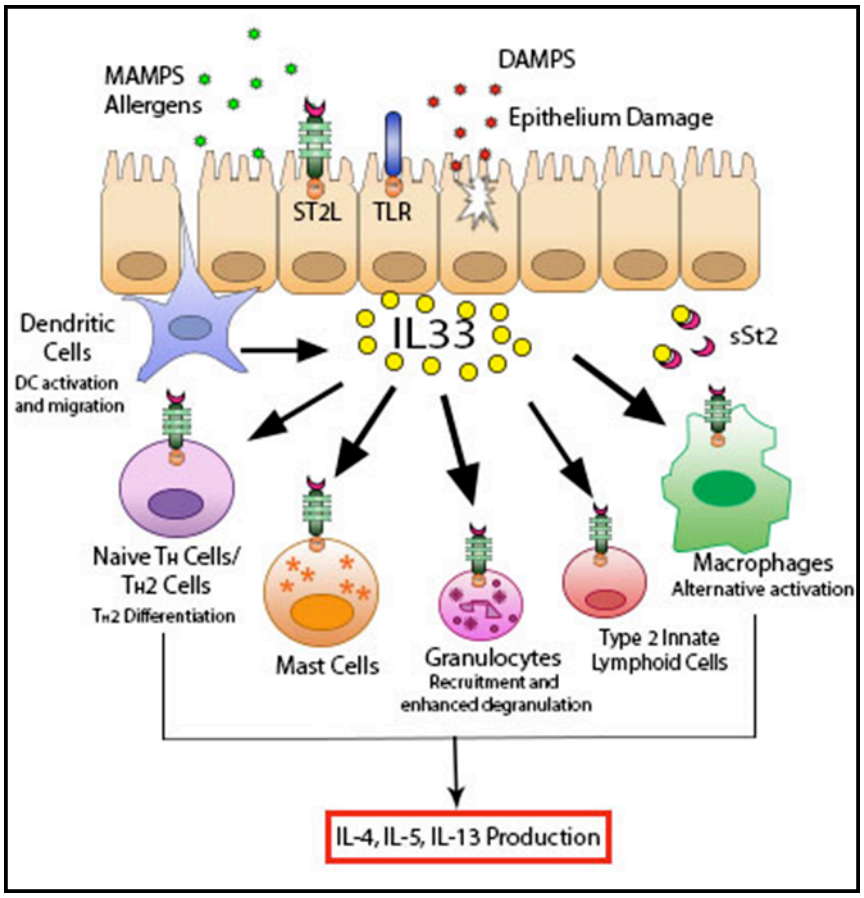

Figure 2: IL-33 signaling pathway in lung. IL-33 is released from airway epithelium in response to various triggers. IL-33 signals through STL on different immune cells to promote inflammation, specifically by IL-5, IL-4, and IL-13. Soluble ST2 binds IL-33 to dampen signaling.

Supressor of Tumorgenicity 2 (ST2) and Interleukin-33

Research examining the pathogenesis of asthma has started to center around a more recently discovered pair in the IL-1 family known as the supressor of tumorigenicity 2 (ST2) and its ligand interleukin-33 (IL-33). The ST2 gene was first discovered in "oncogene or serum-stimulated fibroblasts" (32) and as a result of alternative splicing can have different isoforms, including a soluble form (sST2) and a transmembrane form (ST2L). The ST2L form is strikingly similar to the IL-1R suggesting possible gene duplication. ST2L shares 38% amino acid homology with IL-1R as well as a similar tertiary structure of three Ig-like domains (33). Functionally, ST2L is similar to IL-1R as it also associates with IL-1RAP in when activated by its ligand IL-33 (34). Again, adaptor protein MyD88 forms TIR:TIR domain interactions and recruits several other proteins in order to activate two main pathways, NF-κB and p38 MAPK. Soluble ST2 regulates IL-33 signaling by acting as a decoy receptor. The ST2 gene is ubiquitously expressed in most tissues. In fact, sST2 levels are starting to be used as biomarkers for different conditions such as heart failure (35) and recently ulcerative colitis (36). The membrane bound form, ST2L is expressed on many haematopoietic cells, epithelial cells, endothelial cells, and distinctively has been found on T_H2 cells, but not T_H1 cells (37). In lung epithelial and endothelial cells, T_H2 cytokines increased ST2 expression and improved its function (38).

For many years, ST2 was an orphan receptor until the discovery of its ligand, IL-33, in 2005 (39) using a sequence and homology search. Interleukin-33 is a ~30kDa cytokine, with most similarities to IL-18. Although IL-33 was speculated to be cleaved by caspase-1 of the inflammasome, only the full-length form has been shown to be active

(40). IL-33 has a helix-turn-helix domain (41) and can act as a transcription factor, possibly as a repressor (23). Toll-like receptor signaling has been shown to increase IL-33 mRNA in dendritic cells and mucosal epithelium (42). Protein expression of IL-33 has been shown in fibroblasts, epithelial cells, and endothelial cells in tissues such as the lung and gut (43). In response to virus treatment, alveolar macrophages can also produce IL-33 (44). The mechanisms by which IL-33 is released is still highly debated. IL-33 is thought to be a possible alarmin (45), sensing tissue damage, since it released by necrotic, but not apoptotic cells (40). There is also data suggesting IL-33 can be travel through nuclear pores in membrane bound cytoplasmic vesicles in response to mechanical stress (46). Additionally, airway epithelial cells treated with fungus were shown to release ATP causing downstream secretion of IL-33 (47). Lung structural cells treated with house dust mite had higher secretions of IL-33 when treated with LPS, suggesting TLR4 stimulation plays a role in IL-33 release (48). IL-33 induces powerful inflammatory responses by targeting innate cells, such as mast cells (49), basophils (50), macrophages (51), and dendritic cells (52). IL-33 is also known to highly activate eosinophils and promote eosinophilia, a hallmark of asthma, by increasing cell adhesion and survival (53). IL-33 has also been shown to stimulate non-immune cells to produce pro-inflammatory cytokines such as IL-6 and IL-8 (54).

The ST2/IL-33 axis is a very powerful weapon against infections; however any imbalances can lead to detrimental inflammatory diseases, such as asthma. In infections with *Nippostrongylus Brasilensis* (55) or *Trichuris muris* (56), the administration of IL-33 lead to expulsion of the parasites. Any alterations of ST2 or IL-33 levels have been

shown to have an effect of asthma, proving that this axis plays a central role (Fig.2). In asthmatic patients, sST2 serum levels have been shown to increase and especially so during exacerbations (57). In the mouse antigen challenge asthma models, sST2 is increased in both the lung and serum, suggesting that the host is trying to attenuate IL-33 signaling (58). In airway smooth muscle cells (ASMCs), subjects with asthma had higher IL-33 expression, and IL-33 levels corresponded to the severity of their conditions (59). One of the major ways ST2 and IL-33 impact the development of asthma is by driving the T_H2 immune response. Blocking ST2 with monoclonal antibodies *in vivo* proved to decrease T_H2 responses, although T_H1 responses were unaffected (60). Data using ST2 knockout mice is still conflicting. It has been reported that there is no effect on the T_H2 response (61), and exacerbated response (62), while others show an attenuated response (63). IL-33 can also stimulate naïve T cells to become T_H2 cells (63). In *in vitro* and *in vivo* models, IL-33 was able to drive production of T_H2 cytokines IL-5 and IL-13 (39). Giving exogenous IL-33 to mice induced airway inflammation and airway hyperresponsiveness in a MyD88 dependent manner (64). Recently, the primary target of IL-33 was hypothesized to be a novel innate cell, coined the nuocyte. It is also possible that the nuocyte may be the main source of T_H2 cytokines in asthma (65). GWAS studies have also revealed at least 8 IL33 gene SNPs and 15 ST2 SNPs associated with asthma, highlighting the central role of the ST2/IL33 pathway. ST2 SNPs are thought to cause amino acid substitutions which can change the affinity of IL33/ST2 binding and TIR domain interactions between adaptor proteins including MyD88 (66).

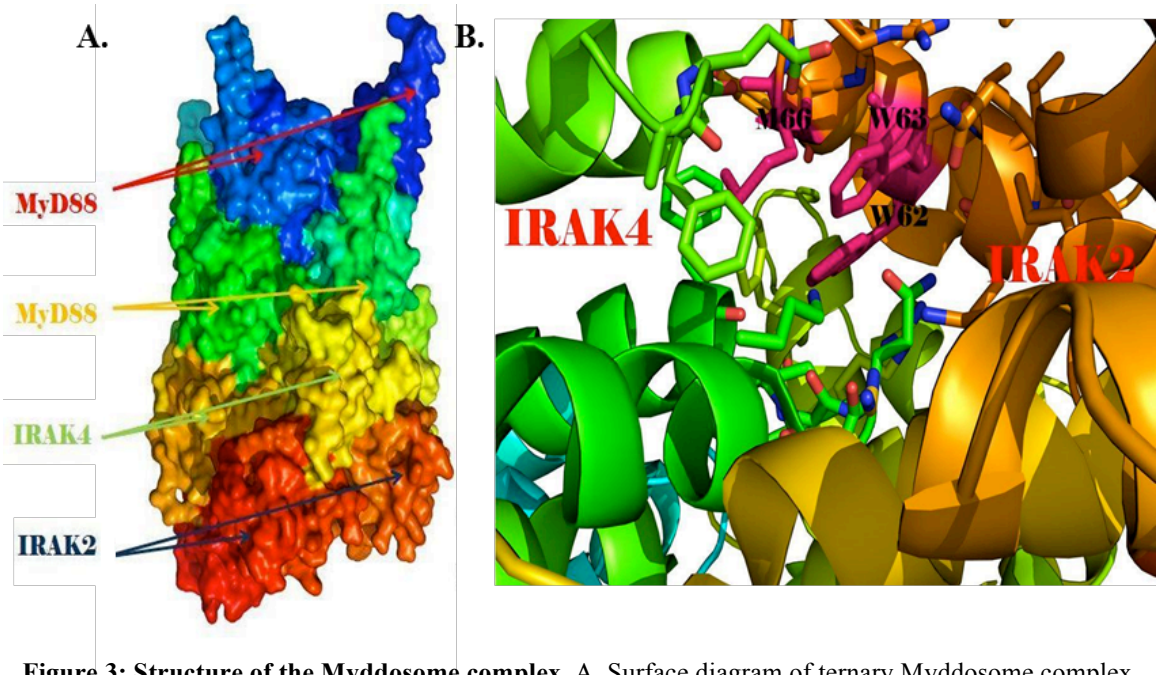

Figure 3: Structure of the Myddosome complex. A. Surface diagram of ternary Myddosome complex B. IRAK B. X-ray structure of IRAK4 and IRAK 2 binding site with key amino acids (M66, W62, and W63) labeled. (Figure modified from Lin, et. al. 2010)

Myddosome

The TIR domain superfamily is the backbone of innate immunity; however, they would be powerless without the adaptor protein MyD88 to carry out the majority of their signals. TLR-3 is one of the only members to use a MyD88-independent pathway (67). Although the necessary protein components of the MyD88 pathway have been well established, the actual structure of the multiprotein complex formed, called the Myddosome, was only defined in 2010 by Lin et al. The foundation of the Myddosome is possible due to two main components of MyD88: a C-terminal TIR domain and an N-terminal death domain (DD). When TLR/IL-1Rs bind ligand, dimerization occurs and the repositioning of their TIR domains recruit adaptor proteins. In the case of MyD88, $TIR_{receptor}$: $TIR_{adaptor}$ associations occur (68) allowing for the death domains of MyD88 to

oligomerize (69). As a result, IRAK-4 is recruited to the complex and stabilized by 3 DD:DD binding interfaces with MyD88. Formation of an IRAK-4 layer allows for the recruitment of IRAK1 or IRAK2 which are also stabilized by DD:DD interactions. X-ray crystallography showed that the completed Myddosome forms a tower structure, stoichiometrically determined to be made of 6 MyD88s, 4 IRAK4s, and 4 IRAK2s (Fig.3A). Once the MyD88-IRAK4-IRAK1/2 complex is complete, phosphorylated IRAK 1/2 is able to form a complex with TRAF 6, causing TRAF 6 ubiquination and downstream activation of the NF-κB pathway and MAPK cascade (70). As a result, the production of T_H2 cytokines IL-4, IL-5, and IL-13 are greatly increased, as well as the critical adhesion molecules ICAM and VCAM (71). In particular, IL-13 has been highly implicated in the pathogenesis of asthma since it has been proven to cause eosinophilia, airway hyperresponsiveness, and mucous production (72). Possible regulatory mechanisms for the Myddosome include IRAK-M, which is thought to act as an inhibitor if TLR/IL-1r signaling by preventing the dissociation of IRAK-1/2 (73). Additionally, it is proposed that IRAK-2 is more necessary for TRAF-6 ubiquination and subsequent activation of NF-κB; whereas, IRAK-1 activates alternative pathways (74). Although much of the MyD88 pathway still remains an enigma, it is clear that targeting the Myddosome has great potential in dampening the deleterious effects of inappropriate TLR/IL-1R signaling, which can lead to the development of asthma.

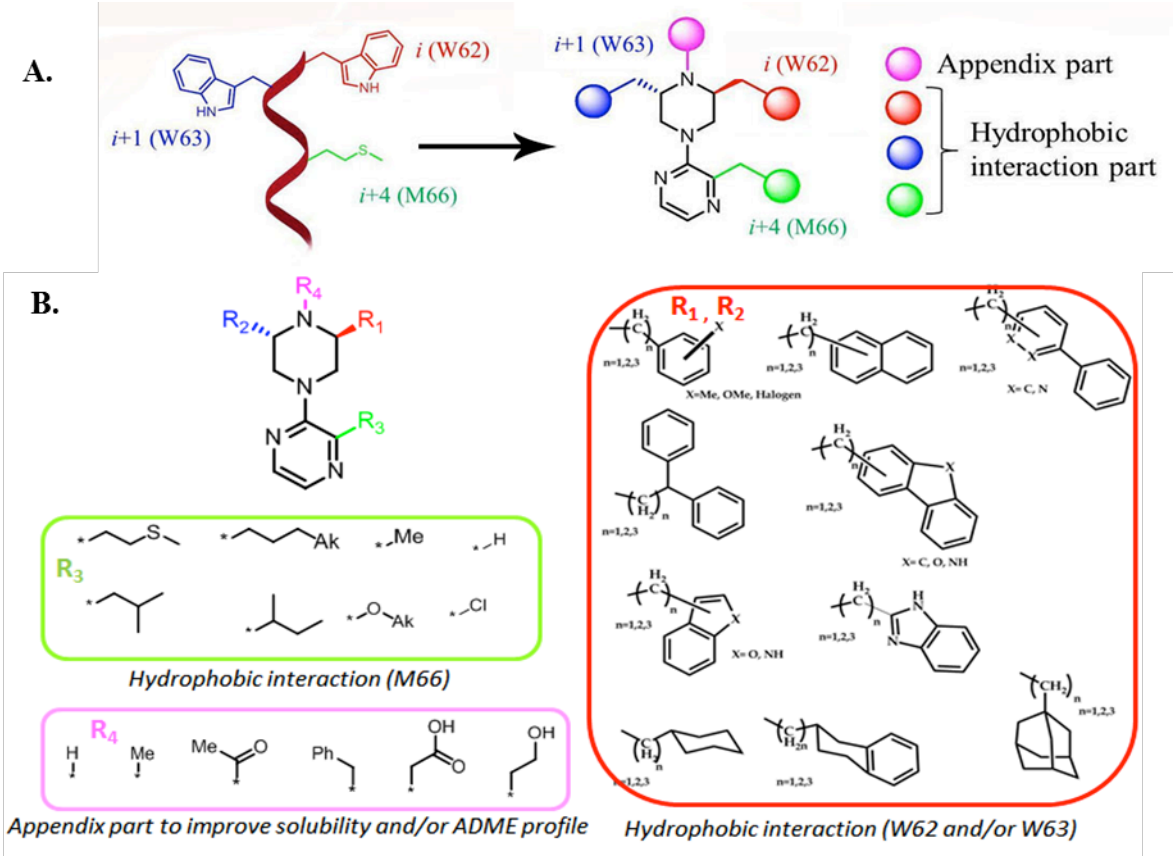

Figure 4: Synthetic route for creating α-helical mimetic library. A. Pyraznyl piperazine scaffold B. Possible groups to form library

Alpha-helical mimetics

The discovery of the Myddosome structure has made it a possible target for blocking TLR/IL1-R signaling. In order for the Myddosome to signal, the MyD88:IRAK4 complex must bind IRAK2. MyD88:IRAK4 and IRAK4:IRAK2 protein-protein interactions have different binding properties, which allow for specific configuration of the Myddosome. A new strategy for developing therapeutic agents has been in the creation of small molecules that can disrupt protein-protein interactions.

Progress has been slow since protein-protein interactions use larger, less defined surface areas for binding compared to very specific binding pockets of enzymes (75). However, much advancement has been made recently, especially in respect to alpha-helical mimetics (76). IRAK4:IRAK2 interact through the alpha-helices of their death domains. Specifically, three amino acids of IRAK2, i.e. W62, W63 and M66 at i, i+1, i+4 of the α-helix are essential for IRAK2-IRAK4 interactions (Fig. 3B). Mutagenesis studies established that two residues (W62 and M66) were critical for signal transduction of MyD88. The Rebek lab at The Scripps Research Institute has used molecular modeling to develop a novel i, i+1, i+4 α-helical mimetic scaffold that resembles the IRAK2 interface in IRAK4:IRAK2 interactions. A pyrazinyl piperazine scaffold allows for the presentation of sidechains in a similar orientation to the i, i+1, i+4 of the α-helix of IRAK2 (Fig. 4A). Additionally, synthesis of mimetics using pyrazinyl piperazine scaffolds is straightforward, results in pure enantiomers, and does not require difficult chiral chromatography. Pyrazine and piperazine rings contain hydrophilic, basic nitrogens which assumedly will make the mimetics water-soluble. Both the pyrazinyl and piperazine rings can be synthesized from amino acids and can be fused together by a simple nucleophilic reaction. A library of alpha-helical mimetics was created by using the pyrazinyl piperazine scaffolds and adding a variety hydrophobic aromatic and alkyl groups. The R1 and R2 groups at the piperazine ring was substituted with phenyl, naphthalene, biphenyl, diphenylmethane, benzimidazole, cyclohexyl, admantane, and various heterocyclic structures. The R3 group that mimics methionine imitated with flexible hydrophobic residues such as normal alkanes, branched alkanes, alkoxyls and alkylhalides (Fig. 4B). Currently, the Rebek lab has synthesized 15 mimetics which have

hydrophobic substituents on the ortho position of the pyrazine ring and both sides of the piperazine ring. These compounds have the potential to competitively bind to IRAK4 of the myddosome complex, preventing downstream activation of pathways such as NF-κB and MAPK. Since most TLR and IL-1R signaling uses Myd88, these compounds are expected to have widespread anti-inflammatory properties, which is promising in targeting asthma.

Precisely how ST2 and IL-33 cause asthma is still unknown. To define these mechanisms, we established asthma-like models in mice and examined how the development of airway hyperresponsiveness and inflammation was altered in various knockout mice. Our studies show that IL-33 is required for both the innate and adaptive immune response in asthma. Interleukin-33 signaling is mediated by the myddosome and this response can be attenuated by an alpha-helical mimetic of IRAK2 derived in the Rebek lab. Further studies of IL-33 role in the immune response and possible inhibitors of this pathway may lead to improved therapies for asthma.

III:

Materials and Methods

Mice

All studies were reviewed, approved and monitored by the VA IACUC. Studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and under AAALAC guidelines. Mouse strains studied included C57BL/6J from Jackson Laboratories (Bar Harbor, ME), RAG1 -/- from Jackson Laboratories (Bar Harbor, ME), MyD88 -/- from Dr. S. Akira (Research Institute for Microbial Disease, Osaka University, Japan), and ST2 -/- from Dr. K. Nakanishi (Hyogo College of Medicine, Japan) originally from Dr. S. Akira (Research Institute for Microbial Disease, Osaka University, Japan). RAG1 -/-, MyD88 -/- and ST2 -/- animals were housed in barrier room facilities in the Veterinary Medical Unit (VA Medical Center, La Jolla, CA).

Compound Delivery

The lead compound, 7004, was dissolved in a solution of 50 DMSO:30 PEG:15 Ethanol:5 Pbs. Osmotic pumps (Alzet 2001) were placed subcutaneously 1 day before the start of the IL-33 protocol (the day before starting intratracheal challenges). The pumps were loaded with 250 µl of the active compound at a concentration of 20 mM or vehicle (DMSO:PEG:Ethanol:PBS). The osmotic pumps delivered 1 µl/h of 7004 (9.5µg/h) or vehicle for 5 days.

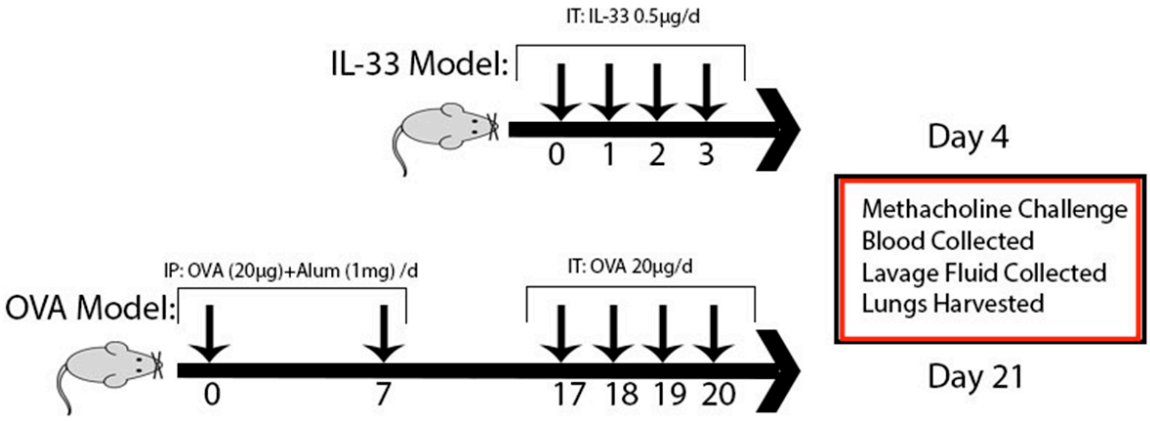

Figure 5: Asthma-like models: A. IL-33 model, mice were challenged with intratracheal installations of mrIL-33 on days 0-3. B. OVA model, mice were immunized intraperitoneally with OVA on day 0 and day 7. Immunized mice were challenged with intratracheal installations of 20 µg of OVA on days 17-20. On day 4 (IL-33 model) or day 21 (OVA model), animals were studied.

IL-33 Model

Animals (approximately 7-12 weeks of age) were treated with the stimulus mrIL-33 using a 4 day protocol developed in our lab. On days 0-3, animals received 0.5 µg mrIL-33 in 50 ml saline by intratracheal instillation under light isoflurane anesthesia using the direct laryngoscopy technique by Hastings (75). On day 4, the animals were studied (Fig. 16).

Ovalbumin Model

Animals (approximately 8 weeks of age) were immunized and sensitized to ovalbumin using a 21 day protocol modified from a previous study (76). On day 0 and day 7, animals were immunized by intraperitoneal injection of 50 µg ovalbumin and 1 mg alum in saline. On days 17-20, animals received 20 mg ovalbumin in 50 µl saline by intratracheal instillation under light isoflurane anesthesia using the direct laryngoscopy technique by Hastings (75). On day 21, animals were studied (Fig. 16).

Measurement of Airway Resistance and Responsiveness

On day 21 for the ovalbumin model or on day 4 for the IL-33 model, animals were anesthetized with 20 mg/kg ketamine and 20 mg/kg of xylazine. Anestheia was induced with 3.5% isoflurane for 1 minute and intubated with a 1 inch 20 gauge IV catheter (Optiva, Johnson & Johnson). Animals were then placed on a computer-controlled small animal ventilator (Flexivent, SCRIEQ, Montreal, Canada) delivering 2% isoflurane continuously. The animals were ventilated quasi-sinusoidally (150 breaths per minute, 6 ml/kg with 3 cm H20 of PEEP). Following paralysis with 0.1 mg/kg of pancuronium bromide, small volume amplitude oscillations at a frequency of 0.9 Hz were applied at a constant volume to the airway opening for 16 seconds and respiratory system resistance (Rrs) was calculated. The animals were then challenged with an ultrasonic aerosol of saline containing 0, 1.5, 3, 6, 12 and 24 mg/ml of methacholine for 10 seconds each. The peak Rrs with each dose was used to generate a dose response curve.

Post-mortem procedures in mice

Under deep anesthesia, the chest was opened with a midline sternotomy. Animals were exsanguinated by collection of blood sample from the heart. The lungs were lavaged three times with 0.5 ml aliquots of saline. The lungs were then partially inflated with air, left atrium excised, right heart cannulated, and lungs perfused with 2 ml saline. The left lung was removed and flash frozen for subsequent studies. The right lung was fixed via the trachea with 0.5 ml of 10% formalin and then placed in 10% formalin for at least 24 hours.

Cell Analysis

Total cell counts were measured by a hemocytometer count using 10 ul of bronchoalveolar lavage fluid (BALF). The BALF was centrifuged at 300xg and

supernatant was flash frozen for immnoassays. The cell pellet was resuspended in saline to 1x106 cells/ml and cytocentrifuged. The cytospins were stained with MayGrunwald Giemsa, and 500 cells counted to determine the differentials.

Histology

Fixed lungs were embedded in paraffin, sectioned (4 μm), and stained with periodic acid-Schiff reagent (PAS). Samples were analyzed using a Nikon 80i brightfield fluorescence microscope interfaced with automated image analysis software. Airway, perivascular, and alveolar/interstitial inflammation were assessed using this system on PAS stained slides.

Multiplex analysis of cytokine expression

Interleukin-1β, Il-2, IL-4, IL-5, IL-10, IL-13, IL-17, IgE, GMCSF, and IFNγ in BALF were measured using a multiplex immunoassay on a Biorad Bio-Plex System. BALF samples were prepped according to the Bio-Rad protocol. The cytokine standard was reconstituted in PBS and diluted to generate an 8-point standard curve for each cytokine. Blanks containing only PBS were also included. The vacuum manifold was calibrated with a standard 96-well plate and a 96-well filter plate was pre-wetted with assay buffer. The multi-plex bead cocktail was added to designated wells and the wells were washed twice with wash buffer. Standards or samples were then added to the plate and incubated at room temperature for 1 hour. After 3 washes the detection antibody was added to the wells and incubated for 30 minutes in the dark at room temperature. Next, the plate was washed three times and streptavidin-PE was added to the plate. After a 10 minute incubation at room temperature the plate was washed 3 times. Assay buffer was added to each well and the plate was read.

In *Vitro* Studies

A. Cell culture

EL4 cells and RAW264.7 cells were purchased from ATCC (Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were transfected with the pGL4.32[luc2P/NF-κB-RE/Hygro] vector (Promega) using FuGene6 transfection reagent (Roche). The pGL4.32 vector has a promoter region containing five copies of an NF-κB response element (NF-κB-RE) fused to a luciferase gene. The stably transfected clonal cell lines were obtained by hygromycin selection at 200 μg/ml.

B. Luciferase Reporter Gene Assay

Stably transfected EL4 cells or RAW264.7 were cultured at 10,000 cells per well and treated with vehicle or experimental compound at a concentration of 100 μM. After 30 minutes, cells were conditioned with 100 ng/ml of IL-33, IL-1β, or LPS for 4 hours. At the end of this incubation period, Steady Glo buffer (Promega) was added to each well and luciferase activity was measured by a luminometer (Promega, Sunnyvale, CA). Fold activation was calculated as the ratio of luciferase activity compared with the control.

C. Immunoprecipitation and Immunoblotting

EL4 cells were cultured at 100,000 cells per well and conditioned with 10 ng/ml of IL-33 and treated with 7004 or vehicle. At 0, 1,5, and 10 minutes cell lysates were collected for each group. Samples were incubated with the IRAK2 antibody at 4C overnight. Next, samples were incubated with magnetic beads for 3 hrs. Samples were kept cold and washed 5 times with lysis buffer, resuspended in Laemmli sample buffer, and loaded on a 4-15% SDS gel. The gel was transferred to nitrocellulose and was incubated in LI-COR Odyssesy blocking buffer for 1hr at room temperature. The blot was then incubated with

IRAK4 antibody at 4C overnight. The blot was washed 5 times and incubated with IRdye 800 for 55 minutes at room temperature. After being washed 5 times, the blot was imaged using the LI-COR Odyssey Imaging System.

II.

Results

Asthma-like Models

Two asthma-like models were developed in C57BL/6 mice, an innate model using IL-33 as a stimulus and an adaptive model using ovalbumin as the antigen for immunization. In the IL-33 model, mice receive 0.5 μg of mrIL-33 through intratracheal instillation for four days. In the ovalbumin model, mice are immunized and challenged with ovalbumin over the course of 21 days. On day 4 for the IL-33 model or day 21 for the ovalbumin model, mice were anesthetized, placed on a computer-controlled ventilator, and paralyzed. Mice were challenged with aerosols of increasing doses of methacholine and airway measurements were taken. Specifically, airway resistance was measured by forced oscillation maneuvers. Afterwards, bronchoalveolar lavage fluid (BALF) was collected and measured for total cell count, cell differentials, and cytokine levels. In IL-33 ($P<.01$) and ovalbumin ($P<.05$) models, mice developed significant airway hyperresponsiveness at the highest methacholine dose compared to controls (Fig.5A). Mice also had very significant increases in cell count and eosinophils compared to controls ($P<.001$) (Fig. 5B). The IL-33 and ovalbumin models had different cytokine increases in the BALF. Mice treated with IL-33 had significantly greater IL-5 ($P<.0001$) and IL-13 ($P<.0001$) compared to ovalbumin immunized and control mice. GMSF levels were increased compared to controls in both models, with greater significance in IL-33 treated mice ($P<.0001$) compared ovalbumin treated mice ($P<.05$) (Fig. 6A). In the ovalbumin model, mice developed increased IL-1β and IL-4 compared to IL-33 treated and control mice (Fig. 6B). Ovalbumin immunized mice also had significantly increased serum IgE levels ($P<.0001$) compared to IL-33 treated and control mice (Fig. 6 C).

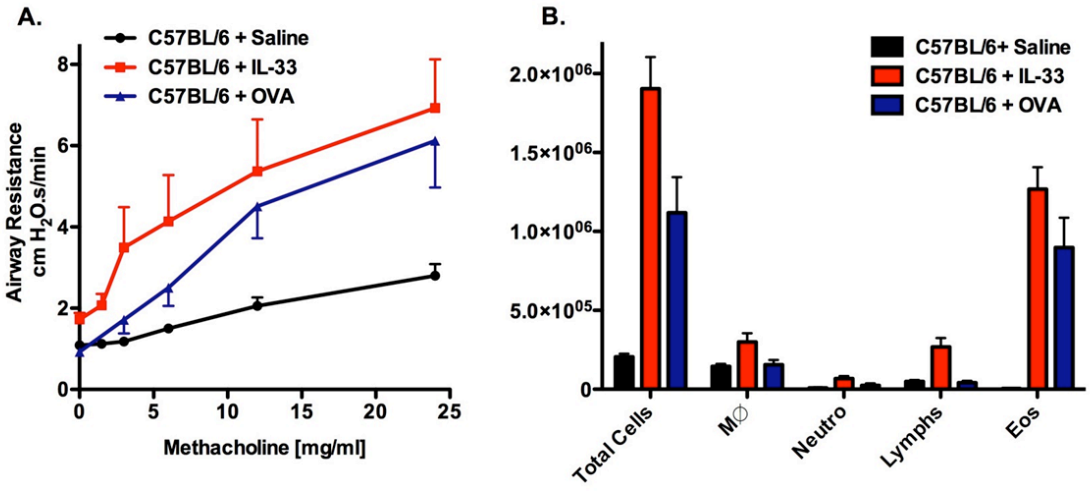

Figure 6: IL-33 induces airway hyperresponsiveness and inflammatory cell infiltrate. Wild-type mice were treated with Saline, IL-33, or OVA. A. Dose response curves to inhaled methacholine. B. Total cell counts of macrophages, neutrophils, lymphocytes, and eosinophils from bronchoalveolar lavage fluid (BALF)

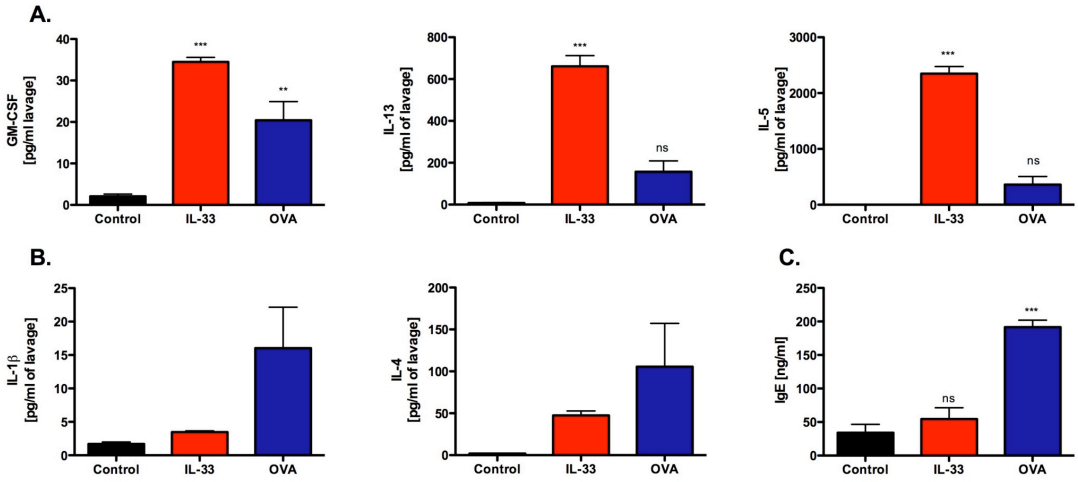

Figure 7: IL-33 induces a different inflammatory cytokine profile than traditional OVA model. Wild-type mice were treated with Saline, IL-33, or OVA. Protein levels of BALF supernatants for A. a) IL-5 b) IL-13, c) GM-CSF B. a) IL-1β, b) IL-4 C. Serum levels of IgE

The IL-33 and OVA asthma-like models were then used in knockout mice to determine the role of IL-33 in the development of AHR and airway inflammation. ST2 knockout mice (C57BL/6 background) were used to test whether the ST2 receptor was the only receptor needed for IL-33 signaling. There were no significant differences in airway hyperresponsiveness in ST2 knockouts treated with IL-33 and controls (Fig. 7A). Total cell count and cell differentials were also not significant compared to controls (Fig.7B). To test whether B cells and T cells are required for IL-33 induced asthma-like phenotypes, RAG1 knockout mice (C57BL/6 background) were given IL-33. RAG1 knockout mice responded similarly to wildtype mice when given IL-33. There were no significant difference AHR (Fig. 8A), total cell count, and eosinophils in IL-33 treated RAG1 knockout mice and IL-33 treated C57BL/6 mice (Fig.8B).

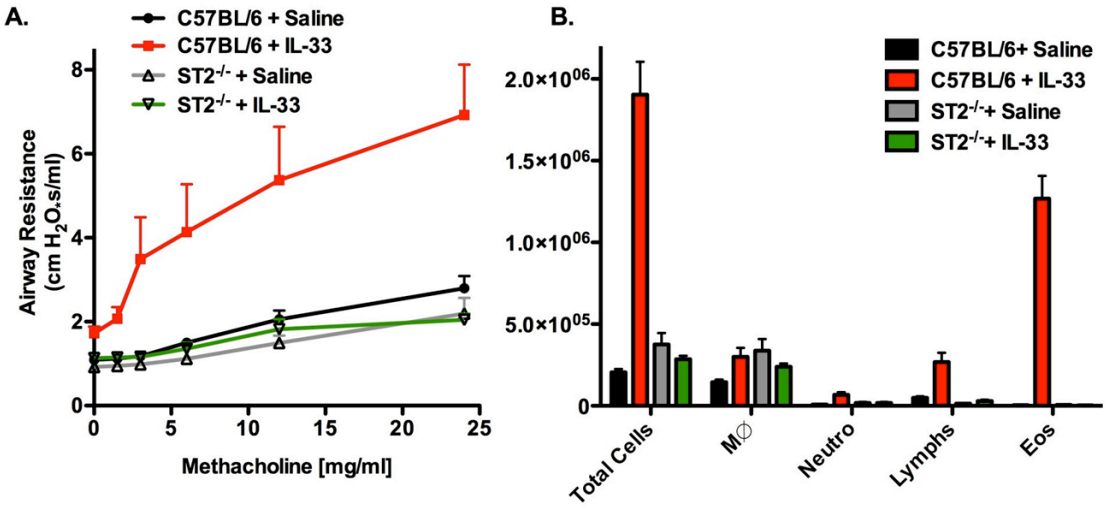

Figure 8: ST2 receptor required for IL-33 induced airway hyperresponsiveness and inflammatory cell infiltrate. Wild-type or ST2-/- mice were treated with Saline or IL-33 A. Dose response curves to inhaled methacholine. B. Total cell counts of macrophages, neutrophils, lymphocytes, and eosinophils from BALF

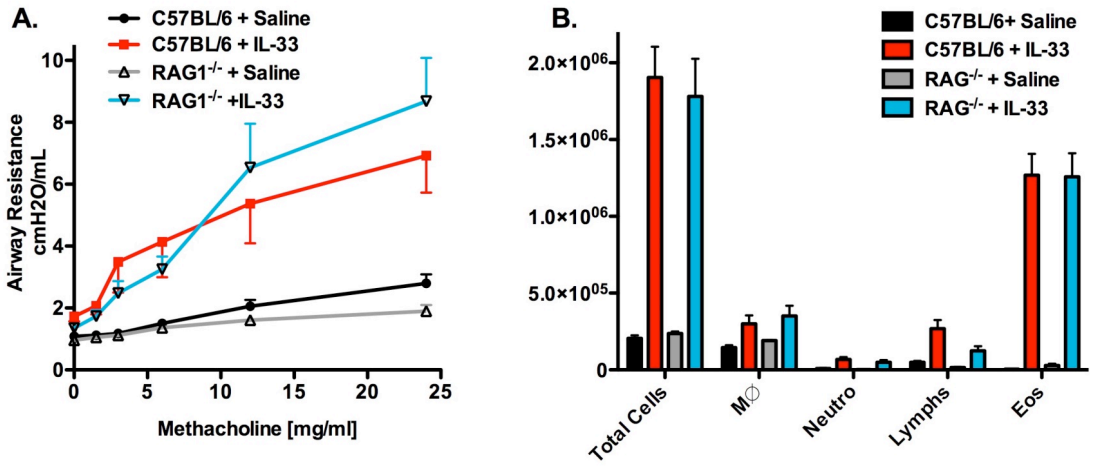

Figure 9: IL-33 induces airway hyperresponsiveness and inflammatory cell infiltrate independent of T cells or B cells. Wild-type and RAG1-/- mice treated with Saline, IL-33, or OVA A. Dose response curves to inhaled methacholine. B. Total cell counts of macrophages, neutrophils, lymphocytes, and eosinophils from BALF

In order to determine whether IL-33 was necessary for the adaptive immune response, IL-33 knockout mice were immunized and challenged with ovalbumin. IL-33 knockout mice had an attenuated response (Fig. 9). MyD88 knockout mice (C57BL/6 background) were used to show that MyD88 is necessary for IL-33 signaling. When given IL-33, MyD88 knockout mice (C57BL/6 background) did not generate an asthma-like phenotype. MyD88 knockouts treated with IL-33 had no significant differences in AHR (Fig. 10A), total cell counts, and cell differentials compared to controls (Fig. 10B).

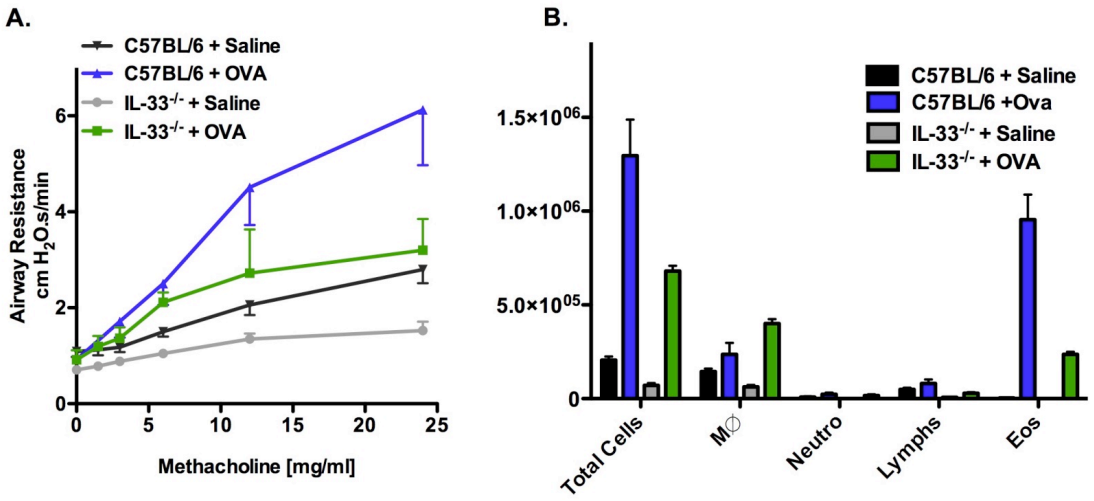

Figure 10: IL-33 required for adaptive (ovalbumin) response. Wild-type or IL-33-/- mice were treated with Saline or OVA A. Dose response curves to inhaled methacholine. B. Total cell counts of macrophages, neutrophils, lymphocytes, and eosinophils from BALF

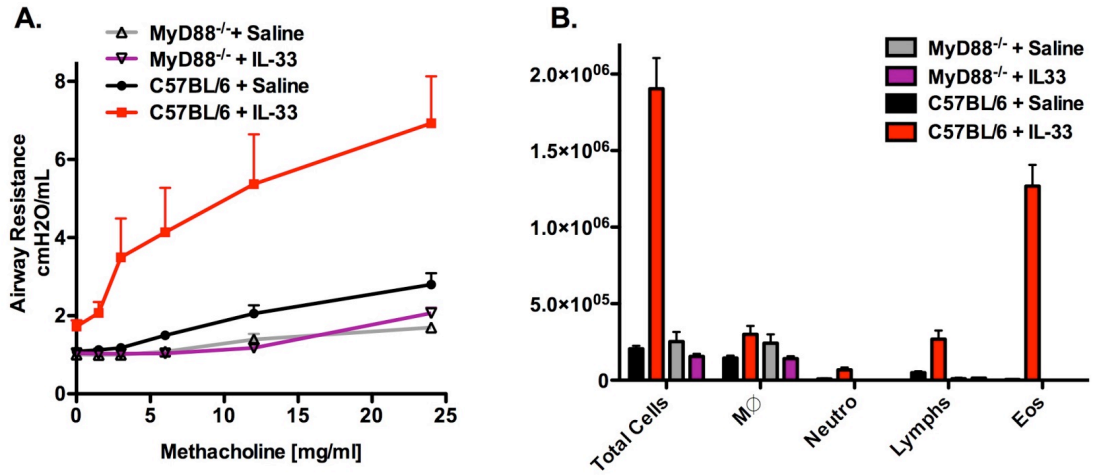

Figure 11: MyD88 required for IL-33 induced airway hyperresponsiveness and inflammatory cell infiltrate. Wild-type C57BL/6 mice were treated with Saline or IL-33 A. Dose response curves to inhaled methacholine. B. Total cell counts of macrophages, neutrophils, lymphocytes, and eosinophils from BALF.

7004 *in vitro*

A library of alpha-helical IRAK 2 mimetics generated by the Rebek lab were tested *in vitro* for effectiveness in blocking IL-33 induced NF-κB activation via the myddosome. *In vitro* studies were done using murine cell lines stably transfected with

the pGL4.32 vector, composed of an NF-κB response element containing promoter fused to a luciferase gene. Cells were treated with compound or vehicle for 30 minutes then stimulated with IL-33 for four hours. Water soluble compounds were dissolved in saline and lipid soluble compounds were dissolved in DMSO. A total of 14 compounds were assayed at 100uM doses on the transfected T lymphocyte cell line EL4 (Fig. 11). Of these compounds, dose response curves of the most effective water soluble or lipid soluble compounds were generated and IC_{50} was calculated (Fig. 12 A,B). The lead compound, 7004, was found to have the lowest IC_{50} at 9.7 μM (Fig. 12 B) and was chosen as the focus for future studies.

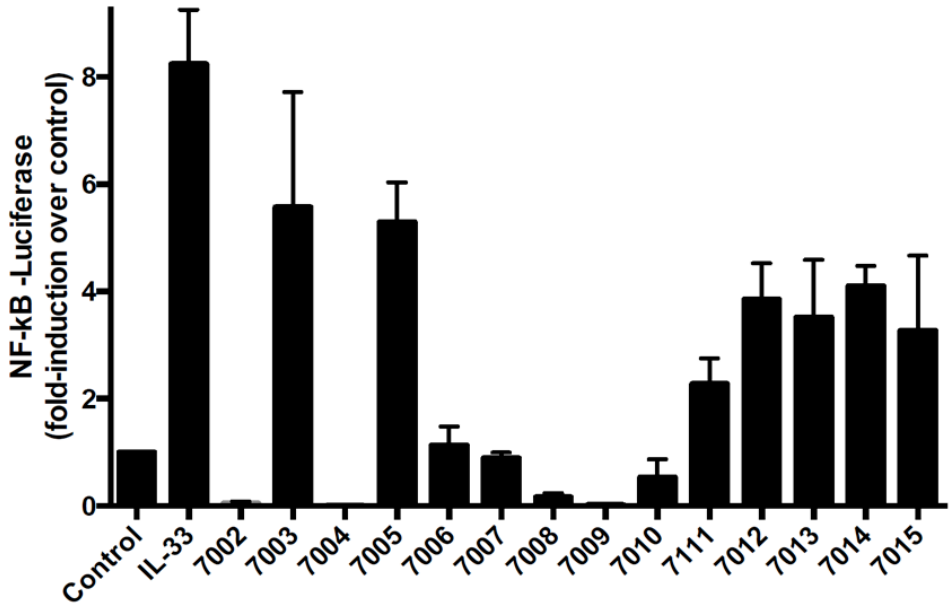

Figure 12: Screening of the α-helical mimetic library. Effect of α-helical mimetics on NF-κB activity in cells treated with IL-33 by luciferase assay. The expression vector pGL4.32 was stably transfected into EL-4 cells. Compounds were screened at 100 μM concentrations.

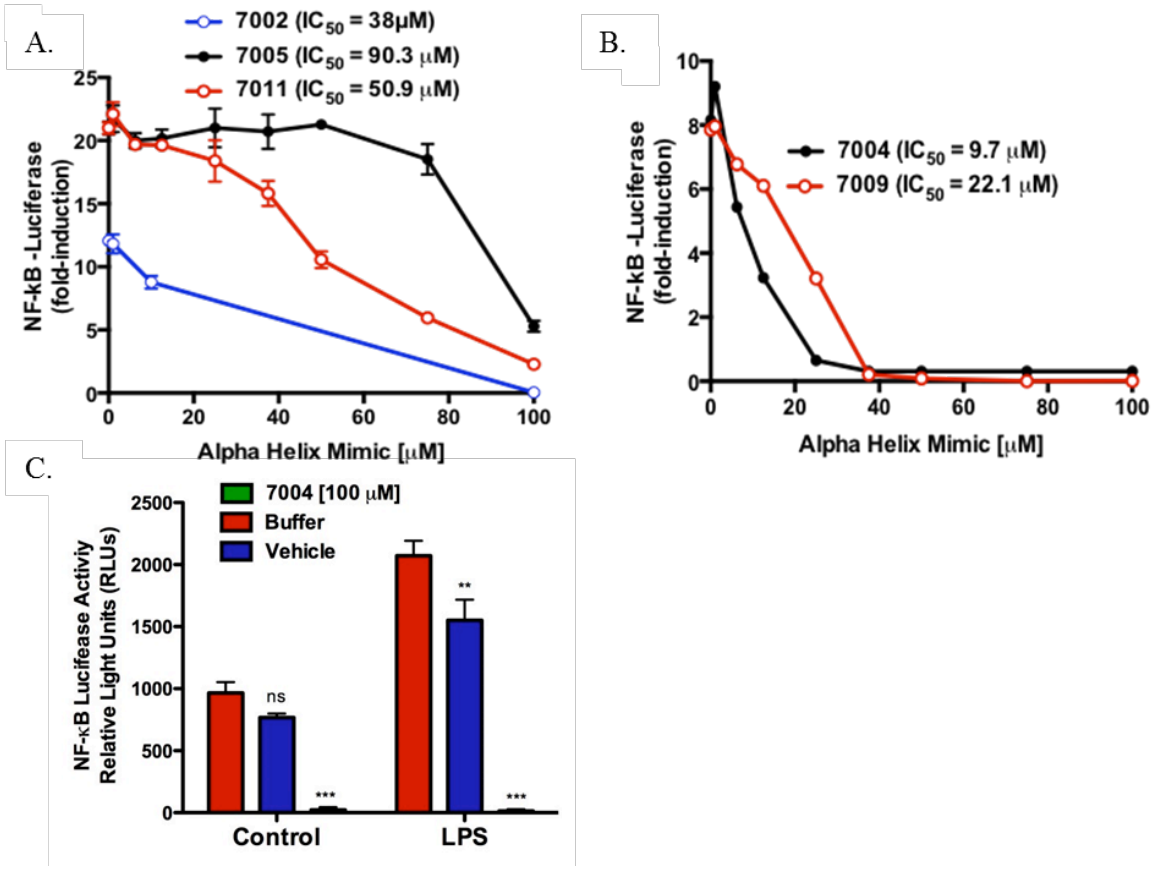

Figure 13: 7004 is the most effective α-helical mimetic in library and silences TIR signaling *in vitro*. A. Dose response curves of water soluble compounds in transfected EL4 cells treated with IL-33. B Dose response curves of lipid soluble compounds in transfected EL4 cells treated with IL-33. C. Transfected RAW 264.7 cells treated with LPS in Buffer, Vehicle, or 7004 at 100uM.

To reduce the toxicity due to vehicle, different solvents were tested to dissolve 7004. A solution of 50% DMSO, 30% Polyethylene glycol, 15% ethanol, and 5% PBS was found to be the least toxic vehicle without reducing the solubility of 7004. The efficacy of 7004 in blocking NF-κB activation was further tested in the transfected macrophage cell line RAW 264.7. At the 100 μM concentration, 7004 was able to significantly silence all NF-κB activation in response to LPS ($P<.001$) and in untreated cells ($P<.001$) compared to vehicle controls. In vitro studies were also done in EL4 cells

to determine if 7004 was silencing NF-κB activation by blocking the formation of the myddosome. In the immunoprecipitation of IRAK2 followed by the immunoblot probing for IRAK4, after 5 minutes and 30 minutes, 7004 caused decreases in IRAK4:IRAK2 binding (Fig. 13).

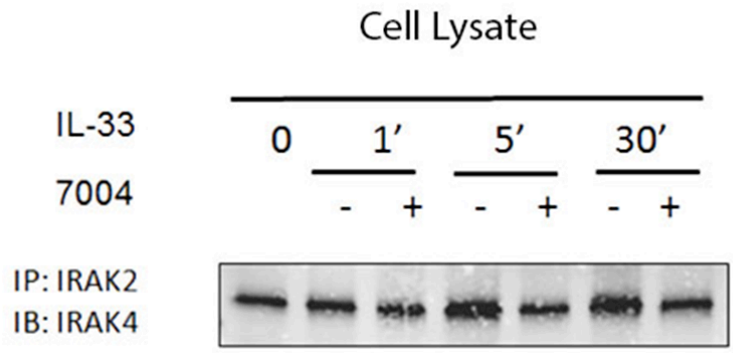

Figure 14: 7004 decreases IRAK4:IRAK2 binding. Immunoprecipitation of IRAK2 followed by an immunoblot probing for IRAK4 in EL-4 cells treated with 7004 or vehicle followed by IL-33.

7004 *in vivo*

The innate and adaptive asthma-like models were then used in C57BL/6 mice to test the effectiveness of 7004 in blocking the development of airway hyperresponsiveness and airway inflammation. An osmotic pump containing 20mM 7004 or vehicle was placed subcutaneously 1 day before intratracheal challenges with IL-33 or OVA. Mice given 7004 did not develop airway hyperresponsiveness in response to IL-33 when compared to controls (Fig. 14) IL-33 treated mice given 7004, also had decreased total cell counts and eosionphils (Fig. 14). When mice were treated with 7004 and given IL-33, airway remodeling was markedly decreased. These mice show less hyperplasia, mucus, airway wall thickening, and cell infilitrate than mice treated with vehicle and given IL-33 (Fig. 15).

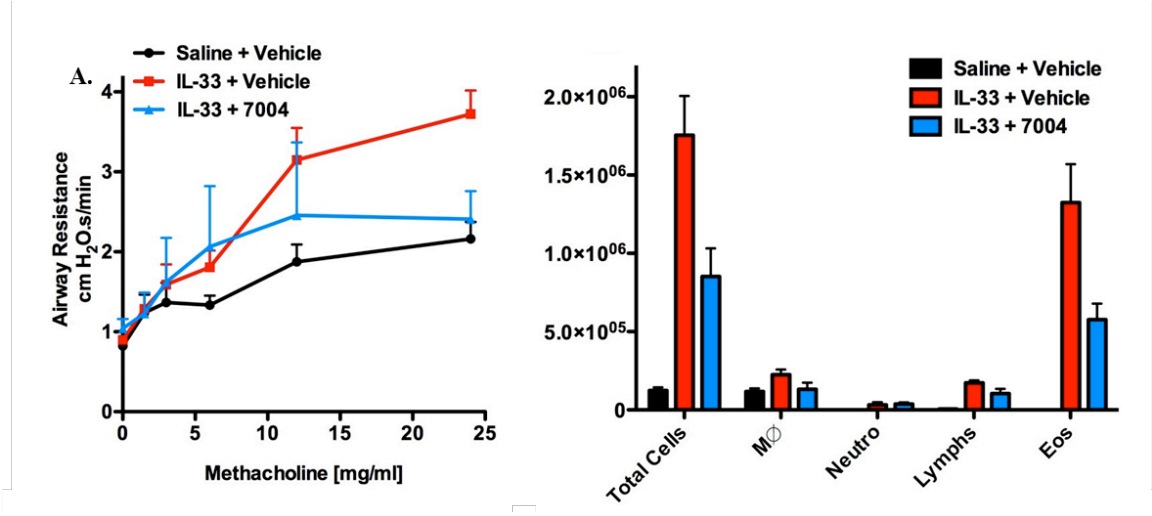

Figure 15: 7004 decreases IL-33 induces airway hyperresponsiveness and inflammatory cell infiltrate *in vivo*. Wild-type mice given 7004 or Vehicle and treated with IL-33 or Saline. Dose response curves to inhaled methacholine. Total cell counts of macrophages, neutrophils, lymphocytes, and eosinophils from BALF. A. IL-33 treated mice.

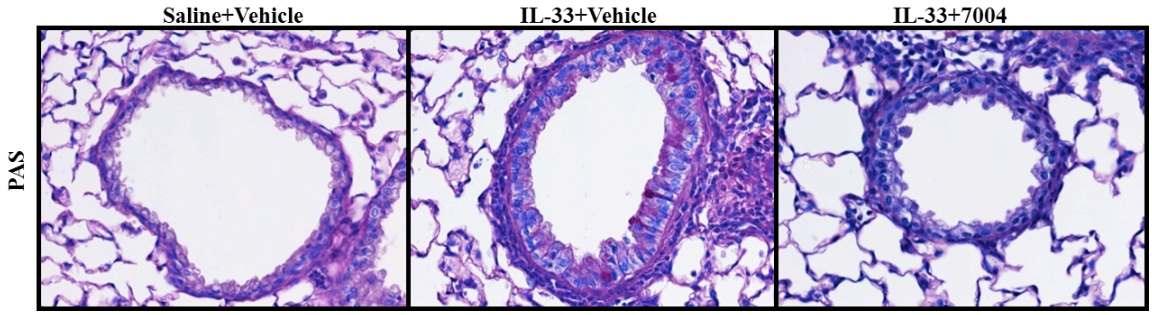

Figure 16: 7004 decreases airway remodeling, mucous secretion, and inflammatory cell infilitrate *in vivo*. Wild-type mice given 7004 or Vehicle and treated with IL-33 or Saline. Lungs were fixed in 10% formalin, embedded, and stained with PAS. Images were taken at 400X.

III.

Discussion

Interleukin-33 is crucial for inducing T_H2 immunity which can have protective or deleterious effects for the host. Both innate and adaptive immune cells are activated by IL-33 to promote inflammation. Its receptor, ST2, uses the adaptor protein MyD88 to carry out the induction of NF-κB and MAPK pathways. In the lungs, IL-33 can start and perpetuate inflammation which may lead to the development of asthma. In particular, the cytokines IL-5 and IL-13 have been shown to cause eosinophilia, airway remodeling, and AHR, which are important hallmarks of asthma. To further study the effects of IL-33, we developed a short term mouse challenge model that uses IL-33 as a stimulus to cause an asthma-like phenotype. We compared our findings to a previously established allergenic asthma model in which mice are immunized and challenged with the antigen ovalbumin (OVA). Since the IL-33 model does not require immunization time and no antigen is used, its response is purely innate. However, the OVA model requires immunization to develop an adaptive response to the ovalbumin. The classical characterizations of asthma, airway hyperresponsiveness and airway inflammation, were apparent in both IL-33 and OVA models. Interestingly, the IL-33 model had a different cytokine profile than the OVA model. In the BALF, IL-33 treated mice had remarkably higher levels of IL-5 and IL-13. This suggests that the source of these cytokines are innate, rather than adaptive cells. It is likely that these cells belong to the subset of newly discovered type-2 innate lymphoid cells, such as nuocytes. On the other hand, OVA models had increased IL-4, which corresponds to the increase in IgE, as IL-4 causes IgE class switching. By utilizing knockout mice, we were able to uncover important roles of IL-33 in asthma. Since ST2 knockout mice were unresponsive to IL-33, we determined that the IL-33 response is receptor mediated and only signals through ST2. Studies in RAG1 knockout

mice proved that mature B cells and T cells were not necessary. However, in IL-33 knockout mice, the OVA model only had an attenuated response. Notably, we concluded that IL-33 does not require the adaptive immune response; however, adaptive immunity is dependent on IL-33 to carry out its inflammatory response. We also determined that IL-33 signaling requires the adaptor protein MyD88 to induce the asthma phenotype. Further papers revealed that MyD88 forms a structure with IRAK4 and IRAK2, called the myddosome, in order to carry out signals from TIR receptors.

The Rebek laboratory at The Scripps Research Institute was able to focus on the protein:protein interactions between IRAK4 and IRAK2 during myddosome binding. They created a library of α-helical mimetics based on the death domains of IRAK2. By inhibiting the complete formation of the myddosome, we predicted that the compound would block IL-33 signaling and other TIR receptor signaling in vitro and in vivo. Screening of the α-helical mimetics showed that many compounds did indeed block IL-33 induced activation of NF-κB. Unfortunately, the most effective compound, 7004, was lipid soluble, which tends to have more vehicle side effects. However, with a low IC50 of 9.7μM, 7004 was chosen as the focus of our studies. By dissolving 7004 in a solution of 50% dimethyl sulfoxide (DMSO), 30% polyethlyene glycol, 15% ethanol, and 5% PBS instead of 100% DMSO, we were able to reduce the toxicity without noticeable changes in effectiveness. Further testing showed that 7004 was also able to block LPS induced NF-κB activity, suggesting its ability to block all Myd88 dependent signaling. An immpunoprecipitation of IRAK2 followed by an immunoblot of IRAK4, showed that 7004 reduced IRAK 2 binding to IRAK4 in IL-33 treated T lymphocyte-like cells. The successful in vitro studies gave us confidence to move to mouse models. We tested the

effectiveness of 7004 in the IL-33 model. Our data revealed that 7004 was able to attenuate AHR and airway inflammation induced by IL-33. Histology showed markedly less airway remodeling, mucus, and cell infiltrate in 7004 treated animals. These preliminary studies show therapeutic potential for α-helical mimetics in inhibiting IL-33 in asthma.

Our studies only begin to shed light on the mechanisms by which IL-33 functions; however, all discoveries are relevant as IL-33 is proving to be involved in a myriad of immune diseases. In the relatively short time that it has been discovered, it has been implicated in asthma, allergy, anaphylaxis, cardiovascular disease, pain, and arthritis (41). An array of inflammatory cells and mediators contribute to the pathogenesis and advancement of asthma, which is why therapies targeting downstream cytokines, such as IL-4 and IL-5, are ineffective. Instead, we show that blocking the entire pathway of IL-33 has the potential to prevent and attenuate disease. In particular, our studies with α-helical mimetics display a possible future in blocking the myddosome to inhibit IL-33 signaling. By improving upon the structure of 7004, we may be able to get better solubility and effectiveness of the compound, thereby increasing its prospects as a future asthma therapy. Further studies can also reveal more targets in the IL-33 pathway and improve strategies to inhibit IL-33 signaling which can help treat asthma and beyond.

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